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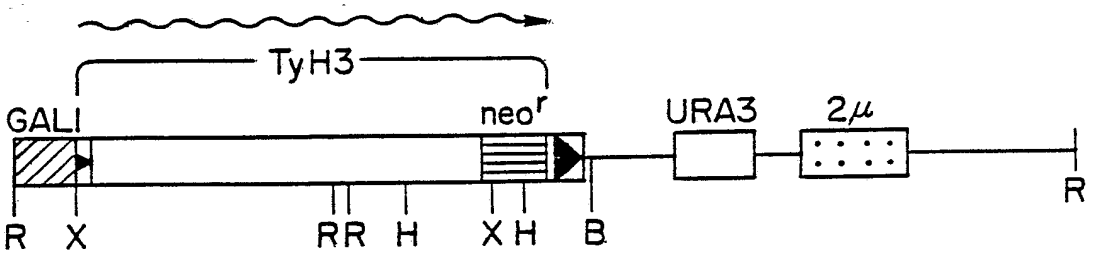

(57) Abstract

A method of amplifying a gene of interest in eukaryotic cells, as well as cells in which amplification can be carried out. According to the present invention, a gene of interest is inserted into a functional retrotransposon, whose transposition or movement in host cell genomic DNA can be controlled by an inducible promoter, to form an amplification cassette. The amplification cassette is introduced into appropriate host cells under non-inducing conditions. Induction of the promoter results in abundant transcription of the amplification cassette, which is accompanied by frequent transposition of the cassette to multiple sites in host genomic DNA. In a preferred embodiment, a gene of interest is inserted into a yeast transposon, or Ty element. The resulting gene-Ty cassette is inserted into a plasmid also carrying the inducible promoter *GAL1*, a 2 micron origin of replication fragment and a *URA3* gene. Yeast cells are transformed with the plasmid under non-inducing conditions (i.e., in glucose-containing media). Cells containing the plasmid are cultured in galactose-containing media, which results in induction of the *GAL1* promoter, abundant transcription of the gene-Ty cassette and its frequent transposition in yeast cell genomic DNA. This results in amplification of the gene of interest. Expression of the increased copy number results in increased production of the encoded protein.

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GENE AMPLIFICATION USING RETROTRANSPOSONS

Description

Background

At present, there is tremendous interest in the potential offered by genetically engineered cells for enhanced or increased expression of genes encoding proteins having, for example, medical applications (e.g., hormones, enzymes, growth factors, other drugs). Production or availability of such materials has historically depended on time consuming, expensive techniques (e.g., extraction from animal tissues, mammalian tissue culture on a large scale, etc.) which often produce small quantities of the protein of interest.

In an effort to increase production of such proteins, scientists have modified both prokaryotic cells (e.g., bacteria) and eukaryotic cells (e.g., mouse, monkey, yeast) by introducing a gene encoding the protein of interest into an appropriate host cell in conjunction with DNA fragments (e.g., a controllable promoter, an inducible origin of replication, a selectable marker) selected to function cooperatively in assisting in selection of

cells containing the gene and in enhancing its expression.

For example, one technique used to increase production of a protein is gene amplification, or production of an increased number of copies of a gene. One application of this technique has been used in increasing dihydrofolate reductase (DHFR) production by chromosomal amplification. Kaufman, R.D. and P.A. Sharp, Journal of Molecular Biology, 159:601-621 (1982).

Presently available methods of increasing gene expression, particularly methods which either rely on gene amplification or are carried out in yeast cells, are limited in their ability to produce the desired result -- increased production of the encoded protein. The number of copies of a gene which can be produced and expressed in yeast cells is limited, for example, by the fact that such strains are unstable and the yield of recombinant product is often lower than expected, based on the copy number of the plasmid.

One approach to modifying yeast cells to produce a protein of interest is to engineer derivatives of the endogenous 2 micron plasmid bearing a selectable nutritional marker (e.g., URA3, or LEU2). Such strains, however, have several disadvantages. First, cells must be grown in minimal medium (which can be more costly) for selection of cells carrying the engineered plasmid to be carried out. Second, the plasmids are often unstable, and are lost by mitotic segregation. This results in

accumulation of plasmid-free cells in the culture, which can, in turn, lead to significant losses of yield, particularly in large fermentor batches. Third, the number of copies per cell of a particular plasmid is limited by the inherent properties of the plasmid and by competition with endogenous 2 micron plasmids.

Disclosure of the Invention

The present invention relates to amplification of a gene of interest in cells. Amplification of a gene of interest can be brought about, through use of the present invention, in eukaryotic cells. According to the method of the present invention, an amplification cassette comprising 1) a gene of interest and 2) a functional retrotransposon, whose transposition or movement in host cell genomic DNA is controllable by an inducible promoter, induction of which results in abundant transcription of the amplification cassette, is introduced into cells by transformation under non-inducing conditions. Induction of the promoter which controls transcription of the amplification cassette results in abundant transcription of the cassette. Abundant transcription is accompanied by frequent transposition of the gene-transposon cassette to multiple sites in host genomic DNA. As a result, the gene-transposon cassette is incorporated into a large number of sites in the genome. Thus, there is an increase in copy number of the gene of interest in the form of stably integrated copies and,

concomitantly, increased production of the encoded protein.

In one embodiment of the invention, a gene of interest is inserted into a yeast transposon, or Ty element. The resulting gene-Ty cassette is inserted into a plasmid which also carries: 1) all or part of an origin of replication whose activation results in replication of the plasmid in high copy number and 2) a gene encoding a selectable marker which allows selection of yeast cells containing (transformed with) the plasmid. In a further embodiment, a gene of interest is inserted into a Ty element and the resulting cassette is introduced into a plasmid which also carries a 2 micron origin of replication fragment; a URA3 gene; and the inducible promoter GAL1. As a result, Ty transcription is placed under the control of the GAL1 promoter, which can be turned on by culturing cells containing the plasmid on galactose-containing media, or shut off by culturing such cells on glucose containing medium. In addition to increasing transcription of the gene-Ty cassette, turning on the GAL1 promoter results in increased transposition of the gene-Ty cassette. Amplification of the gene of interest thus also results and copies of the gene are located at multiple sites in the yeast genome, even after the original plasmid is lost. Expression of the increased number of copies of the gene results in increased production of the encoded protein.

The method of the present invention can be used to cause amplification, or increase in the number of copies of, a gene; integration of the gene copies into yeast genomic DNA; and increased expression of the gene, which in turn results in increased production of the encoded protein.

Brief Description of the Drawings

Figure 1 is a schematic representation of a yeast transposon or Ty element. The open box represents the DNA; the boxed triangles represent LTR (delta) sequences. Represented above the Ty element is the major Ty transcript. Represented below the Ty element are the two open reading frames (ORFs) tya and tyb.

Figure 2 is a schematic representation of a Ty element and other retrotransposons and illustrates the similarities among them. In each case the boxed triangles represent LTR sequences; the open box represents the central coding region; and the boxes below represent the open reading frames. The wavy line above Ty and copia represent RNA.

Figure 3 is a schematic representation of the plasmid pGTyH3-neo (also referred to as pJEF1105). The diagonally hatched box represents the yeast GAL1 promoter; the boxed triangle represents LTR (delta) sequences; the larger open box represents TyH3 internal sequences; the horizontally lined box represents neo^r gene sequences; the smaller open box represents the URA3 gene and the dotted box represents 2 micron circle DNA sequences. The straight

lines shown in the plasmid represent sequences derived from pBR322. The plasmid is circular but is represented in the figure as though it has been linearized by cutting at the EcoRI site which corresponds to the pBR322 EcoRI site. The wavy line above the plasmid represents the Ty-neo cassette transcript. The abbreviations shown below represent the following restriction sites: R, EcoRI; X, XhoI; B, BamHI; and H, HindIII.

Figure 4 is a schematic representation of pGTyH3-neo (pJEF1105) showing the sites (A, B, and C) at which the neo gene was inserted to study transposition in transformants carrying pGTyH3-neo and related plasmids. Sites A and B fall within the open reading frame tyb; site C is outside the open reading frames.

Figure 5 is a Southern blot analysis of cells transformed with pGTyH3-neo.

Figure 6 is a schematic representation of a method for causing repeated cycles of Ty-neo transposition. Figure 6A is a Southern blot showing results of the method represented in Figure 6.

Detailed Description of the Invention

The present invention makes it possible to significantly increase the level of expression of a gene of interest in cells. Through use of the present invention, it is possible to amplify, or increase the number of copies of, a gene of interest in eukaryotic cells (e.g., yeast, mammalian, insect, avian, plant cells) and in viruses. This capability

results from amplification of the gene of interest in a stable form within genomic DNA of cells modified to contain genetic material whose expression within the cells results in transposition in the cell genome of a transposable element-gene of interest construct.

According to the present invention, amplification of a gene of interest is achieved by incorporating into host cell genomic DNA an amplification cassette comprising the gene of interest and a functional transposon. A transposon is a mobile genetic element (DNA sequence) which "moves" within genomic DNA by self duplication, followed by insertion of the new copy (copies) at a location in the genome other than that at which the original (parent) genetic element occurs. Thus, it appears that the original genetic element does not itself move (or hop) within the genome, but, rather, is copied; the new genetic elements are inserted into the genome at new locations. Movement of the transposon to multiple sites in the genome results in concurrent movement of the gene of interest (i.e., both elements of the amplification cassette are duplicated and undergo transposition to multiple sites). Expression of the increased copy number of the gene of interest results in increased production of the encoded material (e.g., a protein). Amplification of a gene of interest can be effected in this manner in a variety of host cell types (e.g., mammalian, viral, yeast, plant, insect cells).

According to the present invention, a gene of interest is amplified in the following manner: An amplification cassette comprising: 1) the gene of interest and 2) a transpositionally functional transposon, such as a yeast transposon, or Ty element, whose movement into host cell genomic DNA can be controlled by an inducible promoter whose induction causes abundant transcription of the amplification cassette, is introduced into an appropriate host. In one embodiment, the amplification cassette is incorporated into a plasmid or other shuttle vector which also carries an inducible promoter, whose induction results in abundant transcription of the gene-Ty amplification cassette. Alternatively, the amplification cassette is introduced into a similar plasmid containing a constitutive (noninducible) promoter whose induction causes abundant transcription of the cassette. A further possibility is the introduction of the amplification cassette into host DNA in integrated form. Induction of the promoter results in abundant transcription of the amplification cassette, frequent transposition of th gene-Ty cassette and, thus, increase in copy number of the gene of interest and the Ty element. Expression of this increased copy number results in increased production of the encoded protein.

In a further embodiment of the present invention, an amplification cassette comprising 1) a gene of interest and 2) a transpositionally functional Ty element whose transposition, or movement into the

yeast (host) cell genome, can be controlled by exogenous signals, is introduced into a plasmid. The plasmid carries a promoter whose induction causes the gene-Ty amplification module to be transcribed abundantly. Preferably, the plasmid also contains an origin of replication or portion thereof whose activation results in replication of the plasmid in high copy number, as well as a gene encoding a selectable marker which makes it possible to identify cells transformed with the plasmid containing the amplification cassette.

In a preferred embodiment of the present invention, a gene of interest is inserted into a Ty element, such as TyH3, whose transposition can be controlled by an external signal, to form an amplification cassette. The amplification cassette is incorporated into a plasmid (e.g., pGTy) which carries: 1) a 2 micron origin of replication fragment; 2) a URA3 gene as a selectable marker; and 3) the inducible (highly controllable) yeast promoter GAL1. The inducible promoter is linked to the Ty element in such a way that the resulting transcript has a 5' end indistinguishable from a normal Ty RNA 5' end. The 2 micron origin of replication fragment is obtained as described below and functions to allow the plasmid to replicate in high copy number. The URA3 gene is a yeast gene which specifies an enzyme in the biosynthetic pathway for uracil and thus makes it possible to select for cells containing the plasmid because it enables ura3$^-$ cells to grow on medium lacking uracil. The

GAL1 promoter is highly controllable; that is, it is "turned off" in cells grown in media containing glucose and "turned on" in cells grown in media containing galactose.

Yeast cells are cultured with plasmids which carry the amplification cassette and the three components described above, under conditions appropriate for transformation to occur. In this embodiment, the co-culturing of cells is carried out in glucose-containing medium (i.e., the GAL1 promoter is off). The resulting cell mixture, which contains transformed and untransformed yeast cells, is cultured in medium which does not contain uracil. Transformants are selected by their ura^+ phenotype (i.e., their ability to grow on the uracil-free medium), which is a well-known selection technique. They are subsequently cultured in galactose-containing media, which results in induction of the GAL1 promoter fused to the Ty element. Induction of the promoter causes frequent transposition of the gene-Ty amplification construct to multiple sites in the yeast genomic DNA. The result of this event is the amplification of the gene-Ty amplification cassette and, thus, availability of an increased number of copies of the gene encoding the protein of interest. Expression of the increased number of gene copies results in increased production of the encoded protein.

The individual components whose presence in transformed yeast cells make it possible to control and increase the copy number of the gene of

interest, and thereby increase production of the encoded protein, are described below, as is their use in increasing production of proteins.

Ty Elements

Analysis of the eukaryotic genome has revealed two broad classes of genes. Genes in the first class are present in low, constant numbers. Genes in this class are immobile and their position on the chromosomes is constant from one member of the species to another; they often occupy the same relative chromosome locations in widely divergent species.

Genes in the second class are present in many copies, which have no characteristic location. Each member of a species may have these mobile genes in different locations. The origin of these elements is unknown, but it is known that they "move" or "hop" within the chromosome. That is, a Ty element initially found at one locus in the chromosome, is reproduced and the new copy is inserted elsewhere in the chromosome (i.e., the initial transposon does not disappear from its initial site when it appears at a new location). One of these mobile genes is the repetitive Ty element of yeast, which represents a family of transposable elements from the yeast Saccharomyces cerevisiae. Such elements are repeated about 35 times per haploid genome and constitute about 4% of the total DNA. Cameron et al., Cell, 16:739-751. (1979).

These 6 kb elements contain a large (approximately 5.2 kb) central region, epsilon, flanked by direct repeats of 334 bp, called LTR or delta elements (Figure 1). Although all Ty elements have this basic structure, they are polymorphic at certain sites Cameron et al., Cell, 16:739-751 (1979).

In Ty elements, there are two overlapping open reading frames (ORF): tya, which appears to be equivalent to the retroviral gag gene and specifies a protein with homology to DNA binding proteins; and tyb, which encodes a protein with homology to the protease, integrase and reverse transcriptase regions of the retroviral pol gene. The gene product of tyb is thought to be synthesized as a tya-tyb fusion protein resulting from a specific frameshift at the end of tya that puts tyb in frame with tya. Clare, J. and P. Farabaugh, Proceedings of the National Academy of Sciences, USA, 82:2829-2833 (1985); Mellor, J. et al., Nature, 313:243-246 (1985).

Ty elements move about the yeast genome by both homologous (i.e., recombinational) and nonhomologous (i.e., transpositional) events. Homologous events (10^{-4}-10^{-5}) are considerably more frequent than nonhomologous events (10^{-7}-10^{-8}). The homologous events involve reciprocal recombination and gene conversion between two Ty elements at different positions in the genome. These homologous recombination events can lead to chromosomal aberrations such as translocations, deletions, and duplications.

The nonhomologous events are transpositions in which a Ty element is inserted into a new chromosomal location unoccupied by a previously existing Ty or delta element. Transposition of a Ty element leads to insertion of a complete Ty flanked by a 5 bp duplication of the target sequence created during transposition. Farabaugh P.J. and G.R. Fink, Nature, 286:352-356 (1980).

The mechanism by which Ty transposition occurs has recently been elucidated by Boeke et al. Boeke, J.D. et al., Cell, 40:491-500 (1985). A Ty element transposes from a DNA copy in one location to a DNA copy in another nonhomologous location through an RNA intermediate. That is, Ty transposition is, in fact, retrotransposition: the flow of sequence information is DNA→RNA→DNA; yeast transposable elements are, thus, also referred to as retrotransposons. This transfer of information from RNA to DNA requires a reverse transcriptase activity. This activity has been found in cells containing induced pGTy plasmids. The activity exhibits pronounced temperature sensitivity; it is almost completely inactive at 37°C, possibly explaining why Ty transposition is temperature sensitive. Garfinkel, D.J. et al., Cell, 42:507-517 (1985).

There is considerable evidence for heterogeneity among Ty elements. These are, for example, two subfamilies of Ty elements: Ty1 and Ty2. Cameron, et.al., Cell, 16:739-751 (1979). Members of the two classes differ in primary sequence in certain coding domains (i.e., in two large areas of substitution),

but the basic structural elements (delta, tya and tyb) are maintained. Warmington, J. R. et.al., Nucleic Acids Research, 13:6679 (1986). The functional difference between the two types of Ty element, if any exists, is unknown. Numerous variant structural forms occur among just the Ty1 elements present in the genome; insertion mutants, deletion mutants and numerous sites of poly morphism for restriction endonuclease cleavage are known to occur. Not all Ty elements are transpositionally functional (i.e. not all are capable of moving within the genome); functional and nonfunctional copies of Ty1 and Ty2 type elements have been found. Either type (Ty1 or Ty2) of Ty element can be used to amplify a gene of interest as described herein, as long as the element selected is transpositionally functional.

The Ty discussed in this embodiment, TyH3, is able to promote high frequency transposition when linked to the GAL1 promoter; other Ty elements may not be able to do so. For example, Ty173 (Simchen et al., Proceeding of the National Academy of Sciences, USA, 81:24231 1984) is nonfunctional when present in such construction, when assayed by a method described below and by Boeke et al., in Cell 1985. Similarly, other Tys carrying deletions, insertions and point mutations may be nonfunctional for transposition. Thus, careful selection of a tranpositionally functional Ty element is essential to amplification of a gene according to the present invention.

Alternatively, in place of a yeast transposable element, another type of transposon functional in yeast cells or other type of host cell into which the amplification construct is inserted can be used in a similar manner. The Ty element and other retrotransposons are represented schematically in Figure 2. Copia, 17.6 and gypsy are from the fruit fly Drosophila melanogaster; IAP is from the mouse. A similar element, bs1, has been found in maize.

For example, the copia, 17.6 or gypsy element of Drosophila melanogaster can be fused to the Drosophila heat promoter in a similar manner to that described for fusion of TyH3 to the Gal1 promoter. The gene of interest could then be introduced into a noncoding segment of the retrotransposon in question. This can be followed by introduction of this amplification cassette into insect tissue cells by transfection (a standard technique) or into whole insects by microinjection of early embryos (i.e., P elements-mediated transformation, also a standard technique). Transposition of the amplification cassette would be induced by heat shock. The latter means of introduction of the amplification cassette would require prior subcloning of the amplification cassette into a P element vector. Rubin, G.M. and Spradling, A.C., Science, 218:348 (1982); Spradling, A.C. and Rubin, G.M., Science, 218:341 (1982).

Inducible Promoters

A promoter site is a specific DNA sequence within a gene which acts as an initiation signal,

recognized by DNA-directed RNA-polymerase, to indicate where transcription to form RNA begins. Inducible promoters are those whose activity can be controlled by external conditions or signals (e.g., temperature change, ion concentration changes, presence or absence of metabolites, such as sugars). GAL1 is a highly controllable yeast promoter whose function can be regulated by the presence or absence of galactose in culture media. That is, the GAL1 promoter is turned on when cells containing the promoter are cultured in galactose-containing media; conversely, the promoter is turned off when media containing glucose is used. Although the present invention as described includes use of the GAL1 promoter, other promoters, whether inducible or non-inducible, can be used in its place. It is important that the promoter used result in increased transcription and reverse transcription of the gene-Ty amplification cassette and transposition of the two elements into the yeast chromosome at multiple sites. For example, in the case of a Ty-neo amplification cassette linked to a GAL1 promoter, it is estimated that the number of transripts produced by the plasmid is about equal to that produced by all of the estimated 30-35 copies chromosomal Ty elements. Expression of the multiple copies of the gene results in increased production of the protein it encodes.

Substitution of the GAL1 promoter for the Ty promoter in the pGTy plasmid used in one embodiment of the present invention results in increased Ty

transcription and increased Ty transposition. The gene of interest inserted into the Ty element is similarly affected: transcription and reverse transcription of the gene occur and the gene of interest is transposed into chromosomal DNA along with the Ty sequences. This results in increased copy number of not only the Ty element, but also the accompanying gene of interest.

Other promoters which can be used include, but are not limited to, a delta promoter (the promoter for Ty itself), ADH promoter, PGK promoter, and PHO promoter. Sentenac A. and Hall, B., In: Molecular Biology of the Yeast Saccharomyces (J. Strathern et al., ed.) 1982.

Two Micron DNA Origin

Most strains of yeast contain a circular DNA sequence, called the 2 micron circle, which replicates autonomously. This plasmid is about 6300 bp in length, occurs at about 50 copies per cell and, like bacterial plasmids, has one origin of replication. Broach J.R. et al., In: Molecular Biology of the Yeast Saccharomyces (J. Strathern et al., ed.) 445-470 (1982).

In the present invention, a 2 micron DNA origin of replication fragment is incorporated into the plasmid which also contains the amplification cassette. The function of this component is to allow the plasmid (and its components) to replicate to high copy number, resulting in increased availability of copies of the gene-Ty cassette for subsequent transcription and transposition. The 2 micron DNA origin is useful because it is known to

allow replication in high copy number. In place of the 2 micron DNA origin of replication, an origin of replication which functions in the host cell (e.g., in the embodiments described above, an origin functional in yeast cells) can be used. For example, an ARS1 or other ARS (autonomously replicating sequence) sequence can be used. The origin selected should be one, however, whose activation results in replication to high copy number of a plasmid containing the gene-Ty amplification cassette.

The URA3 Gene

As described, the present invention preferably makes use of a gene encoding a selectable trait; the gene is incorporated into the amplification cassette-carrying plasmid and allows selection of cells transformed with the plasmid. In this embodiment, the ura3 gene, which encodes an enzyme for uracil biosynthesis is used for this purpose. After transformation of ura3- (e.g. ura3-52) yeast cells with the pGTy plasmid containing the gene-Ty amplification cassette, cells transformed with the plasmid are identified by growing the cell mixture on medium lacking uracil. Cells containing the plasmid (transformants) grow on medium lacking uracil; untransformed cells do not grow. Other genes encoding a selectable marker or conferring a selectable phenotype (e.g., LEU2, TRP1, HIS3, other auxotrophic genes, neo, other drug resistance genes) can be used for this purpose.

Amplification of Genes of Interest to Increase Protein Production

According to the present invention, it is possible to significantly increase production of a protein of interest by incorporating a gene encoding that protein into host DNA as described. As described, the gene is incorporated into yeast genomic DNA, but it is also possible to amplify a gene of interest in other types of host cells, such as viral, mammalian, avian, plant and insect cells. In each of the cell types, the gene of interest is incorporated into the host DNA in combination with a transposable element, which results in movement of the gene of interest. As a result, the gene of interest and the transposable element are amplified and occur in multiple sites in host DNA. Expression of the increased copies results in increased production of the encoded protein.

For example, a gene encoding neomycin phosphotransferase (*neo*^{r} gene) is introduced into yeast cells in conjunction with a Ty element, such as TyH3. In this embodiment, the gene is inserted into the Ty element, which is in turn linked to an inducible promoter, such as *GAL1*. The gene-Ty amplification cassette and the inducible promoter are incorporated into a plasmid which also carries 1) an origin of replication (e.g., a 2 micron DNA origin) whose activation results in replication of the plasmid to high copy number and 2) a gene (e.g., a *ura3* gene) which confers a selectable trait on transformants. Yeast cells are cultured with the

resulting plasmid under conditions appropriate for transformation of the cells. Transformation is carried out using standard yeast transformation techniques, such as lithium acetate (LiAc), as described by Ito et. al. Ito, H. et. al., Journal of Bacteriology, 153:163-168 (1983). Transformants contain the URA3 gene and, thus, can be selected on the basis of growth on medium lacking uracil and isolated.

URA$^+$ cells are then cultured under conditions appropriate for induction or activation of the promoter (e.g., in galactose-containing media if GAL1 is used). As a result, increased transcription, reverse transcription and transposition, of the amplification cassette occurs. The neo^r gene is then located in many sites in the yeast genome, along with the Ty element. Expression of the increased number of neo^r genes results in yeast cells which are resistant to the neomycin analog, G418. If the Gal1 promoter is used, cells containing (transformed with) the plasmid can be exposed to (grown on) galactose-containing media more than once in order to achieve higher copy numbers than result from a single exposure to galactose. Similarly, if other than the Gal1 promoter is used, serial or multiple culturing under conditions appropriate for induction or activation of the promoter can be carried out. This will result in higher copy number of the gene of interest.

The cells remain resistant even when the pGTyneo plasmid is segregated off (segregants are

Ura$^-$), thus proving that the neo^r genes now located in the yeast genomic DNA are expressed. The number of copies present in transformants was assessed using Southern blotting; 2-15 copies of the Ty-neo cassette were shown to occur per genome.

Expression of the neo gene (with a bacterial promoter) and the yeast TRP1 gene (with its own yeast promoter) in yeast cells has shown that these foreign genes are expressed sufficiently to produce, respectively, G418 resistance and a Trp$^+$ phenotype. This level of expression is achieved in spite of the fact that the passenger genes are "embedded" within a Ty element, which upon integration into the chromosome is apparently itself transcribed. Such a situation may not be ideal for optimal transcription of the passenger gene. It may be possible to increase further the expression of the passenger gene by building into the U3 region of the pGTy plasmid to be used a mutation which would render the Ty's own (LTR) promoter inactive once the gene-Ty cassette has transposed into the yeast genome. Such a mutation has been found. K. Durbin, 1985 Cornell University PhD thesis. It might also be possible to fuse the gene of interest to any one of the available yeast promoters (forming an expression cassette) and then insert the gene-promoter combination into the Bg1II site located near the 3' end of the Ty element. This would allow efficient expression of genes which lack promoters which function in yeast.

It has been shown that induction of pGTyH3 and related plasmids results in the formation of virus-like particles (VLPs) which are thought to be an intermediate in Ty transposition. Garfinkel, D.J. et.al., Cell, 42:507-517 (1985); Mellor, J. et.al., Nature, 313:243-246 (1985). This raises the possibility that there is a limit to the size of the gene of interest which can be efficiently transposed in conjunction with the Ty element. However, the neo sequence which has been transposed (Example 1) is 1 Kb long and is transposed about as efficiently as a 40 bp lac0 marker. This suggests that if there is a limit to the length of the gene of interest to be amplified, it is considerably greater than 1 kb.

Using the present invention, it is possible to increase the copy number of a gene of interest and, as a result of expression of the increased number of genes, increase production of the encoded protein. The gene of interest can be a gene which is: 1) normally present and normally expressed at biologically significant levels in cells into which the amplification cassette is introduced; 2) normally present in but not normally expressed at biologically significant levels in cells into which the amplification cassette is introduced; or 3) is not normally present in cells into which the amplification cassette is introduced, alone or in combination.

Such genes, which are also free of non-yeast introns and free of a yeast transcriptional terminator sequence, can be inserted into a transposable

element and amplified in the manner described above. cDNA clones of most genes of interest to be amplified according to the present invention are available and intron-free genes or portions thereof are available for incorporation into the amplification cassette.

Transposition of a gene-Ty element amplification cassette can be achieved in cells other than yeast cells (e.g., other yeast, insect cells, avian cells, mammalian cells, plant cells). An approach similar to that described for yeast cells can be used. In this case, the Ty element is fused to an appropriate promoter derived from the organism to be used as host.

This invention is further illustrated by the following examples, which are not to be seen as limiting in any way.

Example 1 Construction of pGTyH3-neo Plasmid.

pGTyH3-neo (also designated pJEF1105) (Figure 3) was constructed by inserting a 1 Kb BamHI fragment encoding neomycin phosphotransferase from plasmid pGH54 (Joyce et al., Journal of Bacteriology, 158:636, 1984) into a plasmid pGTyH3 which had been partially digested with Bg1II. Plasmid pGTyH3 was constructed as described by Boeke et al., in Cell, 40:491-500 (1985), the teachings of which are incorporated herein by reference. Several recombinant plasmids resulted from the ligation; these plasmids carry the neo fragment at each of the three BG1II sites and in both orientations. (Figure

4) pJEF1105 carries the neo fragment in the same transcriptional orientation as TyH3 in the 3' BglII site. pJEF1105 has been deposited with the American Type Culture Collection (Rockville, MD) under deposit number 67247. It had previously been shown that it is possible to insert genetic information into this site without disruption of transposition. Boeke, J.D. et. al., Cell, 40:491-500 (1985). The pJEF1105 plasmid was transformed into various yeast strains, including BWG1-7a, by selecting for Ura+ colonies.

Example 2 Assessing Transposition Competency of pGTyH3-neo Plasmid

Yeast transformants containing pJEF1105 and some of the related plasmids described above (all transformants studied were Ura+ and G418 resistant as a result of the presence of the plasmids) were examined for transposition competency. The structures of the related plasmids differed from that of pJEF1105 in both the site of insertion of the neo gene into TyH3 (site A, B or C) and the orientation of the neo gene (+, indicating the same transciptional orientation as TyH3; -, indicating transcriptional orientation opposite to that of TyH3). Sites A and B are within the open reading frame tyb; Site C is outside the open reading frames. (Figure 4)

Assessment of transformation competency was carried out in the following way. The cells were plated to form single colonies on SC$^-$ura plates containing 2% galactose as the carbon source for 5 days at 22°C (shown to be the optimal regimen for obtaining transposition of lac0-marked Ty elements

into chromosomal DNA). Randomly selected colonies were streaked to SC-ura glucose plates (to select for plasmid-containing cells) and allowed to grow into colonies at 30°C. The plasmids were then segregated from the cells by growth on YPD (rich) medium. Plasmid-free cells were identified by their Ura^- phenotype. Results of this work are shown in Table 1.

TABLE 1 Assessment of Transposition Competency of Yeast Transformants

Transformant Number	Plasmid Number	Structure of plasmid: Insertion site of neo gene	Structure of plasmid: Orientation of neo gene	Carbon Source	# $Ura^-/G418^r$ colonies	# $Ura^-/G418^s$ colonies
JB512	pJEF1103	B	+	GAL	0/12	12/12
JB516	pJEF1105 (pGTyH3-neo)	C	+	GAL	10/12	2/12
JB516	pJEF1105	C	+	GLU	0/11	11/11
JB518	pJEF1106	B	-	GAL	0/12	12/12
JB515	pJEF1104	A	-	GAL	0/12	12/12

A, B and C in the table refer to the sites at which the neo gene was inserted in the Ty element. See Figure 4.

80-100% of the pJEF1105-transformed cells which went through this procedure were found to be resistant to G418. In contrast, the untransformed strain, and pJEF1105 transformants which were not exposed to galactose failed to show any G418 resistance following loss of the plasmid. Moreover, insertion of the neo fragment at either of the other two BglII sites within the pGTyH3 plasmid (which would lead to frameshift mutations in the Ty-encoded gene products) resulted in plasmids which, unlike pJEF1105, were unable to support transposition of the Ty-neo cassette into the chromosome (Figure 4).

The pJEF1105 plasmid-free segregants which showed G418 resistance were then examined directly to see whether the neo gene had been incorporated into chromosomal DNA. Chromosomal DNA was prepared from the plasmid-free segregants and digested with the restriction enzyme EcoRI. The resulting DNA fragments were separated by electrophoresis on a 0.6% agarose gel and transferred to nitrocellulose by the method of Southern. The presence of multiple copies of the neo gene in 7 such plasmid-free segregants (lanes 1-7) was demonstrated by hybridization of a radioactively labeled probe made from the pGH54 BamHI (neo gene) fragment mentioned above to the Southern blot. No hybridization of the radioactively labeled probe to the control strain (lane +) DNA was observed (Figure 5). The pattern of bands hybridizing was different for DNA derived from different colonies taken from the galactose plate; this is expected if the Ty-neo cassette transposed

essentially at random into chromosomal DNA (because EcoRI sites are distributed randomly in DNA). Thus, these results are in agreement with those obtained when a 40 bp synthetic lac0 fragment was used as a marker earlier with pGTyH3-lac0, where pGTyH3 was marked with a 40bp synthetic lac0 fragment. Boeke, J.D. et al., Cell, 40:491-500 (1985).

Transposition of the Ty-neo cassette into the target plasmid pAB100 was also observed. Boeke, J.D. et. al., Cell 40:491-500 (1985). The Ty-neo cassette showed no evidence of rearrangements or of behaving differently from an unmarked or lac0-marked Ty.

Example 3 Assessment of the Number of Copies of Ty-neo Introduced into Transformants

In BWG1-7a cells exposed to galactose only once for 5 days at 22°C, an average of 3.6 copies of Ty-neo were integrated into the cell genome. However, much higher copy numbers were achieved by putting the transformants through multiple cycles of growth on galactose. A method of causing repeated cycles of Ty transposition is represented in Figure 6. After having been put through five cycles of growth on galactose, BWG1-7a transformants carrying pGTyH3-neo showed no apparent loss of fitness, as determined by fact these cells showed a normal or near normal growth cycle. Cells which have been through five cycles of this process contain, on the average, 14.4 copies of the Ty-neo cassette. Each cycle typically produces a pattern of Ty-neo

insertions which differs from the pattern seen in DNA made from the previous cycle by the addition of an average of 3-4 new copies. (Figure 6A) Passage through additional cycles is likely to result in a further increase in copy number of the cassette. It is conservatively estimated that forty or more copies can be incorporated into yeast genomic DNA by this approach. Mating of a and alpha haploid strains (a and alpha are the two mating types of yeast) which have each undergone the amplification process would double the copy number in the resulting diploid strain (i.e., a product of the mating between the "a" and the "alpha" haploid strains).

Example 4 Amplification of TRP1 gene

The Saccharomyces cerevisiae TRP1 gene was inserted into pGTyH3, forming plasmid pGTy-TRP1, using techniques similar to those described above. This construct appears to work as well as the pGTyH3-neo construct in that it produces multiple copies of a Ty-TRP1 cassette in the yeast cell genome. Prior to insertion of TRP1 into pGTyH3, the sequence of TRP1 was removed. It is possible that the presence of a strong transcriptional terminator within the "passenger gene" might destroy the ability of the Ty-passenger gene cassette to transpose because Ty transposes through an RNA intermediate in a retrovirus-like mode. Truncation of the Ty-passenger gene transcript might render it incompetent for transposition, because the repeated "R" region normally found in both ends of the Ty trans-

cript would be lacking. This region is essential for proper reverse transcription. Varmus, H.E. and Sawnstram, R., In: RNA Tumor Viruses, (2d ed.) (1984); Gilboa, E. et. al., Cell, 18:93-100 (1979). Proper neo transcription is likely to be essential for proper transposition.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. Cells having stably integrated into genomic DNA modified transposable elements, said modified transposable elements comprising a transpositionally functional transposable element and a gene of interest.

2. Cells of Claim 1 in which said transpositionally functional transposable element is a retrotransposon and said gene of interest is inserted into said retrotransposon.

3. Cells of Claim 2 in which the retrotransposon is a yeast retrotransposon.

4. Yeast cells having stably integrated into genomic DNA modified transposable elements, each of said elements comprising a functional yeast retrotransposon having inserted therein a gene of interest.

5. A yeast cell of Claim 4 in which said functional yeast retrotransposon is TyH3 and said gene of interest is inserted therein at an unessential site.

6. A yeast cell of Claim 5 in which said gene of interest is inserted at the 3' BglII site of TyH3.

7. A plasmid comprising:
   a. an amplification cassette, said cassette comprising a transposon, having inserted therein a gene of interest, transposition of said transposon being controlled by an inducible promoter;
   b. an inducible promoter, whose induction controls transposition of said transposon, said promoter being linked to said transposon;
   c. a gene conferring a selectable trait upon cells containing said gene; and
   d. an origin of replication, or fragment thereof, activation of replication at said origin resulting in replication of the plasmid to high copy number.

8. A plasmid of Claim 7 in which the transposon is a yeast transposon, the inducible promoter is GAL1 and the origin of replication fragment is a 2 micon DNA origin fragment.

9. A plasmid comprising:
   a. a TyH3 transposon having inserted therein, at a nonessential site, a gene of interest, transposition of said TyH3 transposon being controlled by a Gal1 promoter;
   b. a GAL1 promoter, said GAL1 promoter being linked to said TyH3 transposon;
   c. a URA3 gene; and
   d. a 2 micron DNA origin of replication, or protion thereof.

10. A method of amplifying a gene of interest, comprising culturing cells, said cells comprising a transposable element having inserted therein said gene of interest, said transposable element occurring at multiple sites within genomic DNA of said cells.

11. A method of amplifying a gene of interest, comprising the steps of:

a. inserting said gene of interest into a functional transposable element, transposition of said element being controlled by an inducible promoter;

b. linking said functional transposable element to an inducible promoter, said inducible promoter being capable of controlling transposition of said transposable element, under conditions at which the inducible promoter is not induced;

c. introducing said functional transposable element linked to said inducible promoter in b) into an appropriate host cell; and

d. culturing cells produced in c) under conditions appropriate for induction of said inducible promoter.

12. A method of amplifying a gene of interest, comprising the steps of:

a. inserting said gene of interest into a functional yeast transposon, transposition of said transposon being controlled by an inducible promoter, to form an amplification cassette;

b. introducing said amplification cassette into a plasmid, said plasmid comprising:

1. an inducible promoter, induction of said promoter controlling transposition of said yeast transposon;

2. a gene conferring a selectable trait on cells containing said gene; and

3. an origin of replication fragment, induction of replication at said origin causing said plasmid to replicate in high copy number;

c. co-culturing an appropriate host cell and the plasmid resulting from step b), under conditions appropriate for transformation of said host cell with said plasmid;

d. isolating host cells transformed in step c);

e. culturing said isolated host cells under conditions appropriate for induction of said inducible promoter,

to cause transposition of said yeast transposon having the gene of interest inserted therein into genomic DNA of said host cells.

13. A method of amplifying a gene of interest, comprising the steps of:

a. inserting said gene of interest into a functional yeast transposon, transposition of said transposon being controlled by a GAL1 promoter, to form an amplification cassette;

b. inserting said amplification cassette into a plasmid comprising:

1. a 2 micron DNA origin of replication, or fragment thereof;

2. a URA3 gene; and

3. a GAL1 promoter,

linking said functional yeast transposon to said GAL1 promoter, to form an amplification cassette-carrying plasmid;

c. culturing said amplification cassette carrying-plasmid with yeast cells under conditions appropriate for transformation of said yeast cells with said amplification cassette-carrying plasmid;

d. isolating yeast cells containing said amplification cassette-carrying plasmid;

e. culturing cells isolated in d) in galactose-containing media,

to allow transposition of said amplification cassette into yeast genomic DNA.

14. A method of Claim 13 in which said functional yeast transposon is TyH3 and said plasmid is PGTy.

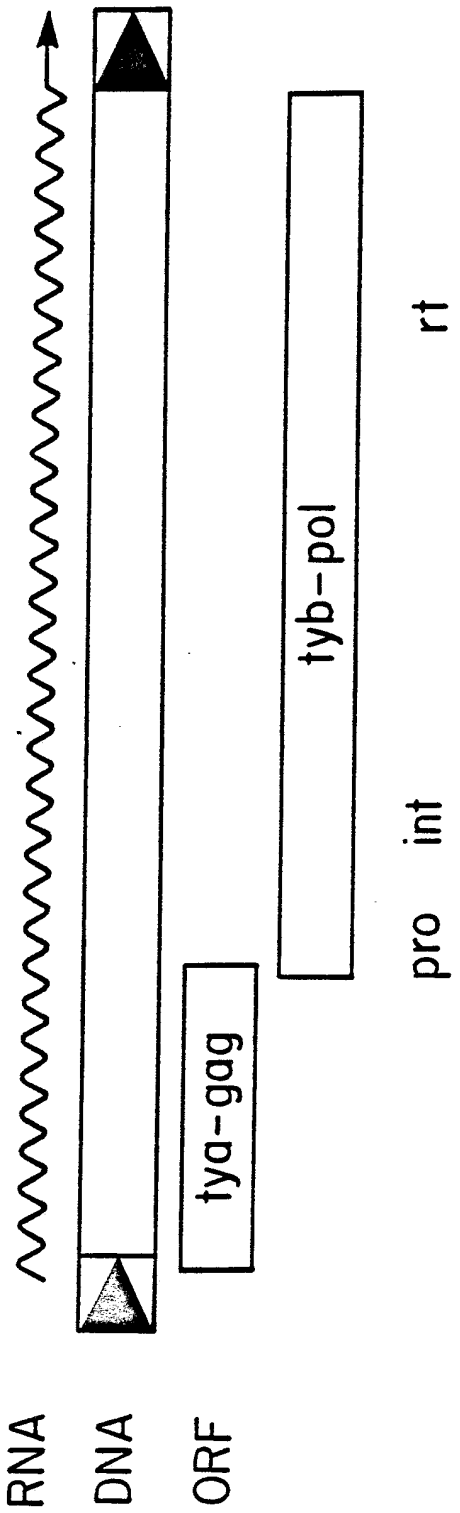
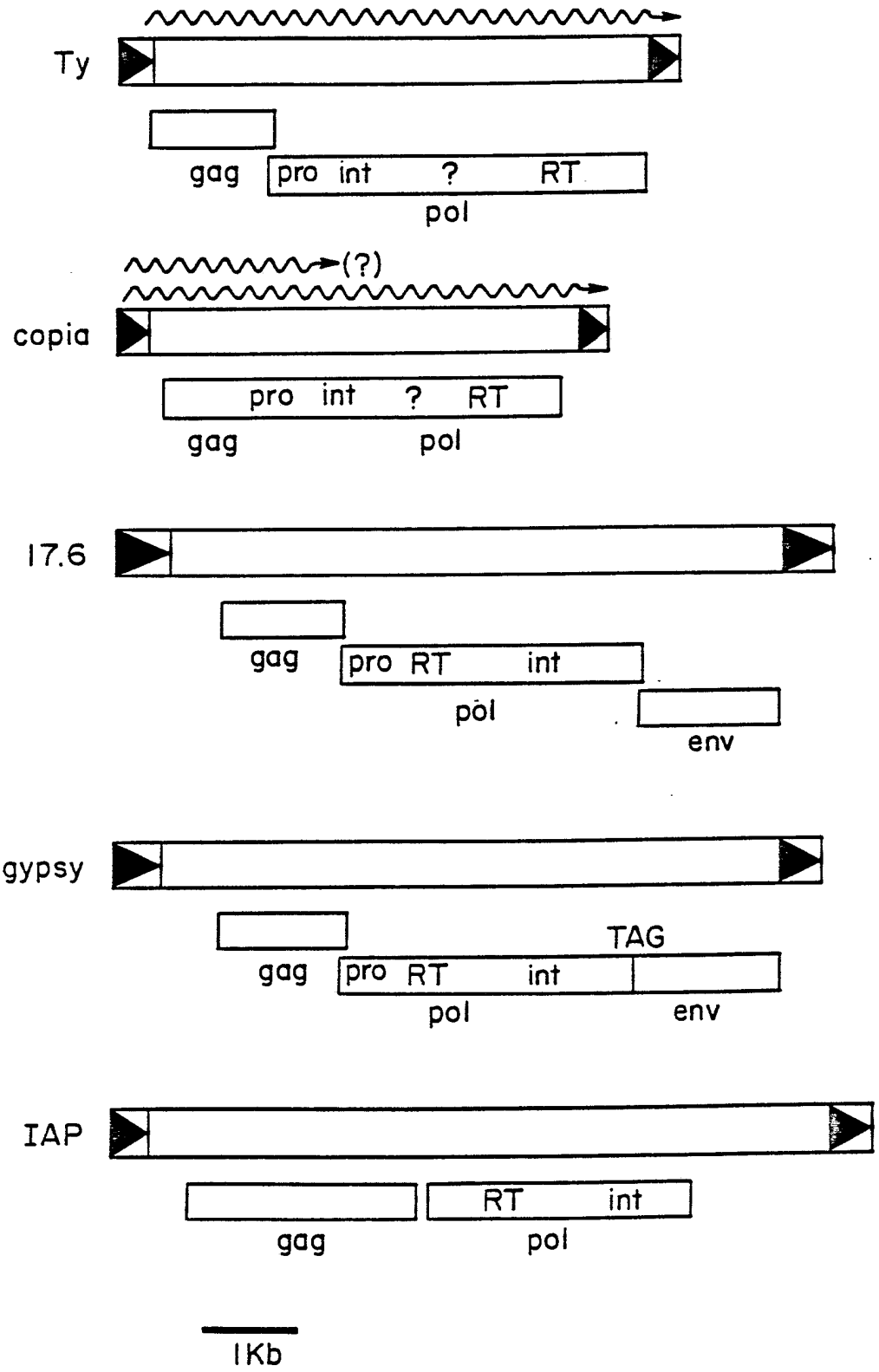


Fig. 1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pro int ? RT
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pro int ? RT
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17.6
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gag
pol
1Kb

Fig. 2

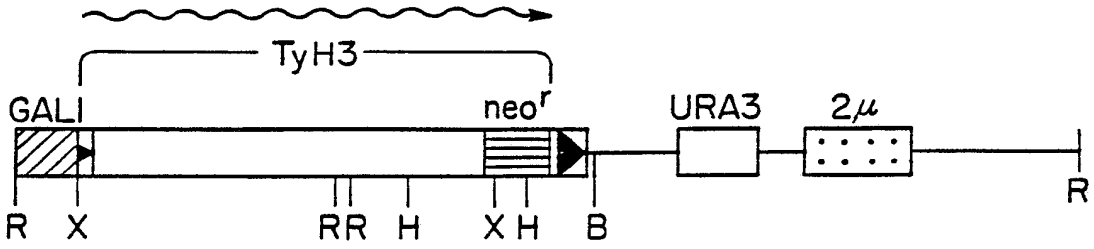
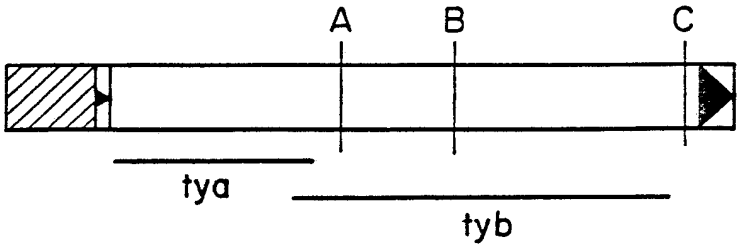


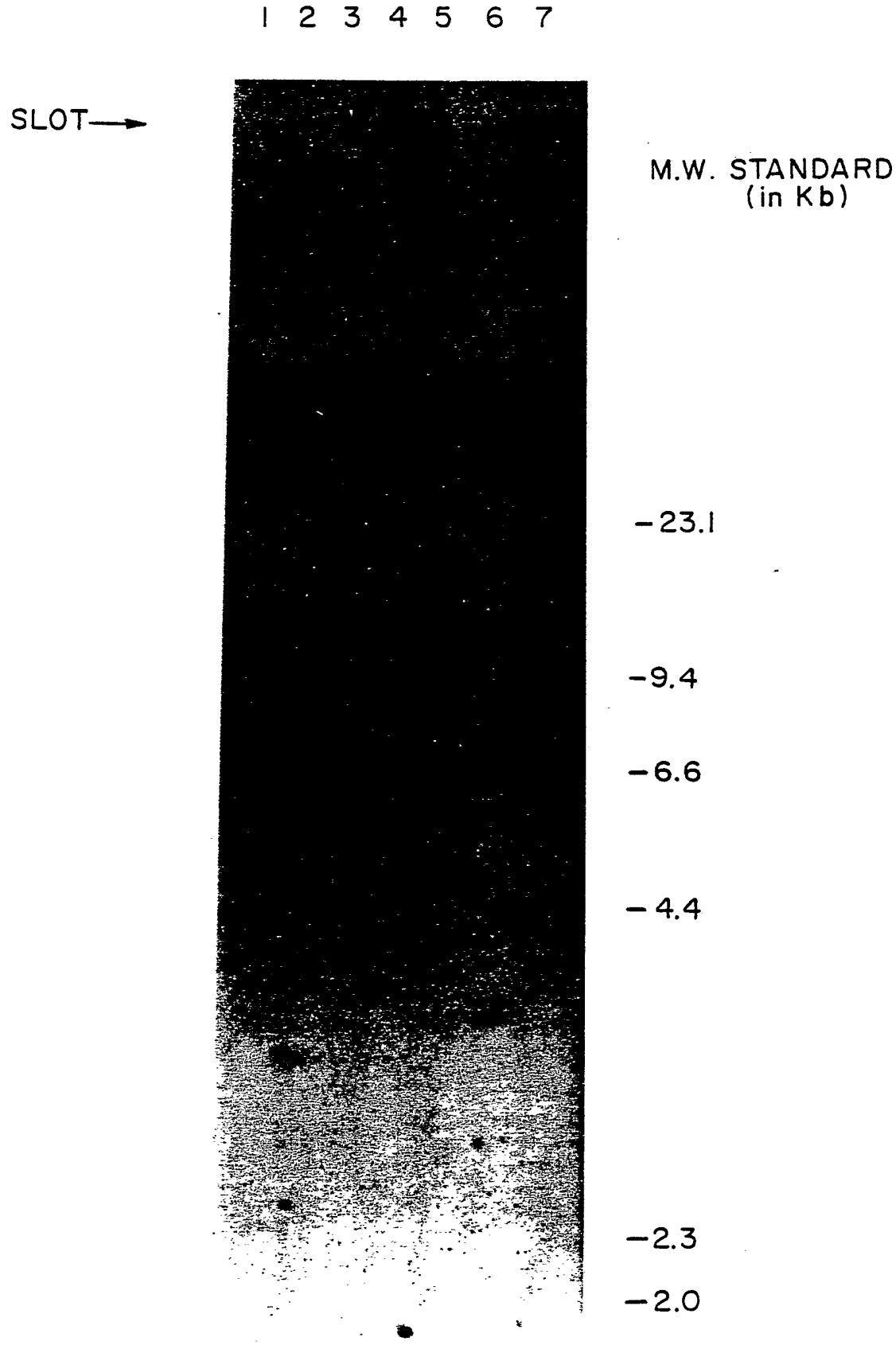
Fig. 3



Bg I II SITES

Fig. 4

FIG.5



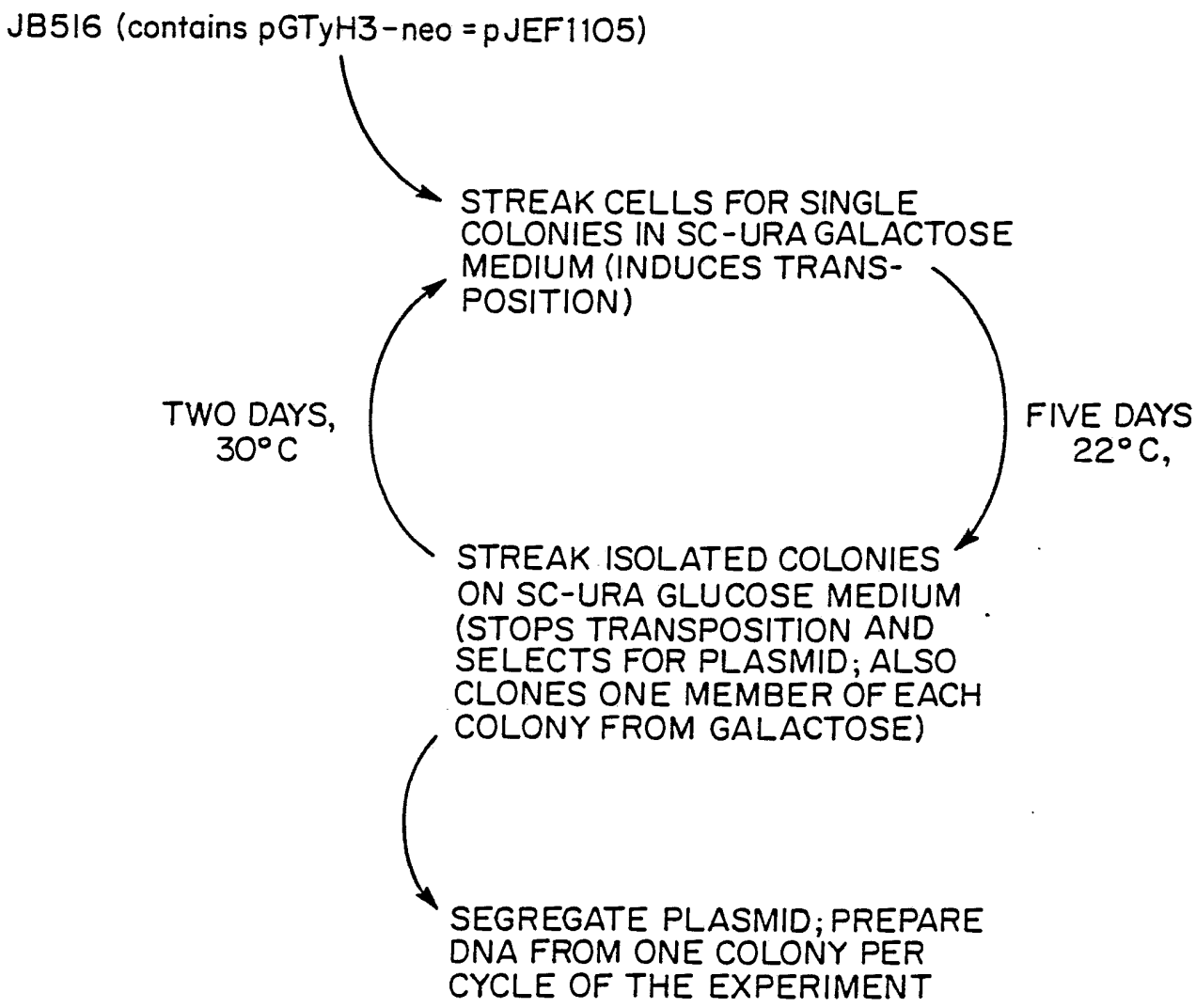
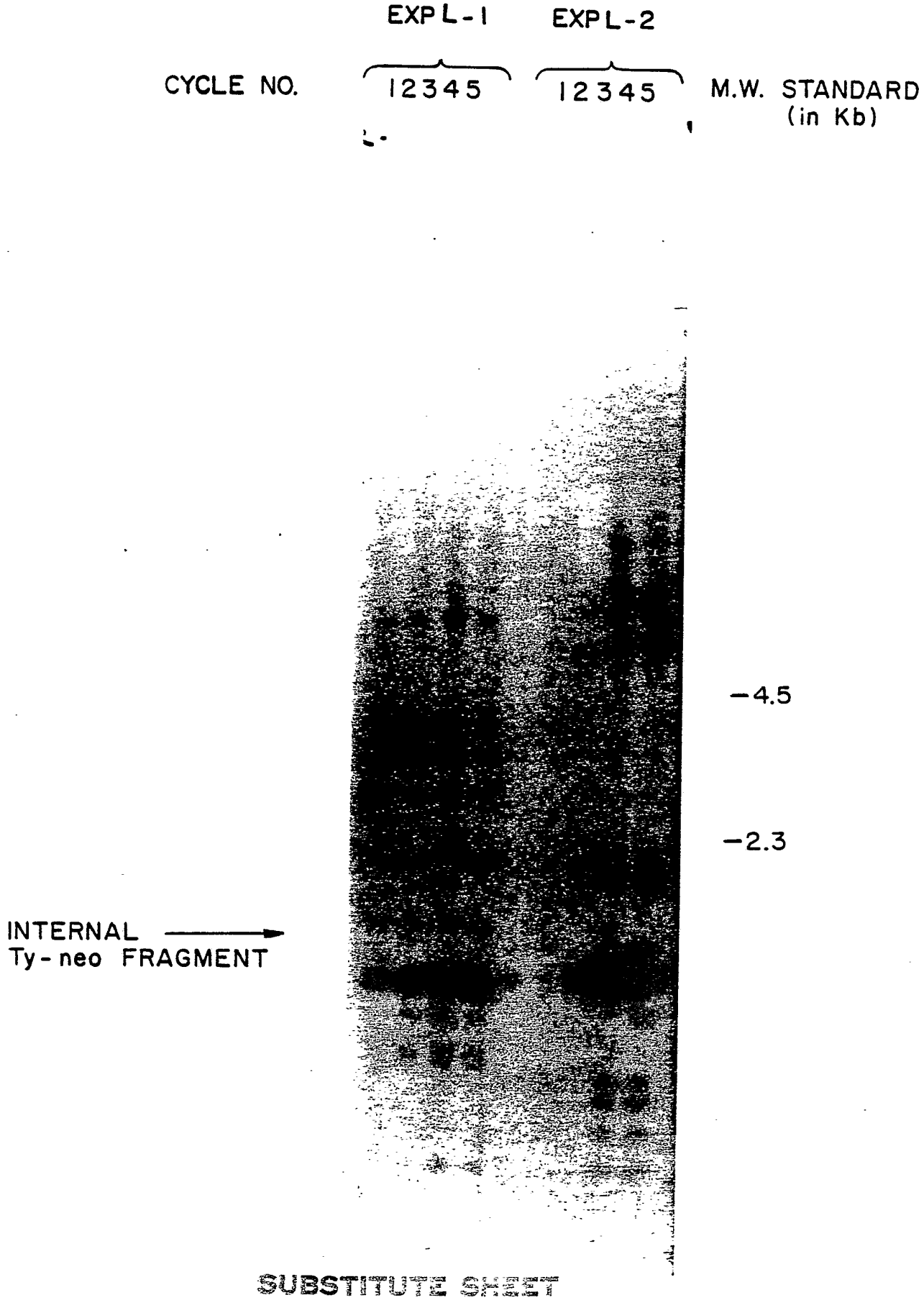

JB516 (contains pGTyH3-neo = pJEF1105)
STREAK CELLS FOR SINGLE COLONIES IN SC-URA GALACTOSE MEDIUM (INDUCES TRANS-POSITION)
FIVE DAYS 22° C,
STREAK ISOLATED COLONIES ON SC-URA GLUCOSE MEDIUM (STOPS TRANSPOSITION AND SELECTS FOR PLASMID; ALSO CLONES ONE MEMBER OF EACH COLONY FROM GALACTOSE)
TWO DAYS, 30° C
SEGREGATE PLASMID; PREPARE DNA FROM ONE COLONY PER CYCLE OF THE EXPERIMENT

Fig. 6

FIG. 6A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EXP L-2
CYCLE NO.
12345
12345
M.W. STANDARD (in Kb)
-4.5
-2.3
INTERNAL
Ty-neo FRAGMENT

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 87/02788

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 1/18

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Nature, volume 313, 17 January 1985, J. Mellor et al.: "A retrovirus-like strategy for expression of a fusion protein encoded by yeast transposon Ty1", pages 243-246 see page 245 --	1-4,10
X	Proc. Natl. Acad. Sci. USA, volume 82, May 1985, J. Clare et al.: "Nucleotide sequence of a yeast Ty element: evidence for an unusual mechanism of gene expression" pages 2829-2833 see the whole article --	1-4,10
A	Trends in Genetics, volume 2, no. 5, May 1986, Elsevier Science Publishers B.V. (Amsterdam, NL), G.R. Fink et al.: "The mechanism and consequences of retrotransposition", pages 118-123 see the whole article, especially figure 3 --------	1-9

* Special categories of cited documents: [10]

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th January 1988	04 MAR 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN